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ULTRACENTRIFUGE STUDIES OF PROTEINS IN MIXTURES OF PHENOL, ACETIC ACID AND WATER

P. E. STANLEY, A. C. JENNINGS and D. J. D. NICHOLAS

Department of Agricultural Biochemistry, Waite Agricultural Research Institute, The University of Adelaide, South Australia

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Abstract—Ultracentrifuge studies have been made of the following proteins dissolved in mixtures of phenol, acetic acid and water: crystalline samples of bovine serum albumin, α-chymotrypsin, insulin and ribonuclease and the proteins of three wheat flours and a dough prepared from one of them. The weight average molecular weights of α-chymotrypsin, insulin and ribonuclease in these solvents were similar to those calculated from their amino acid compositions. The weight average molecular weight obtained for bovine serum albumin, however, in the phenol solvent was about 17,000 as compared with the commonly accepted value of about 69,000, determined in dilute aqueous buffers. The material in aqueous extracts of wheat flours and dough showed rather high apparent molecular weights but when these extracts were dialysed into phenol–acetic acid–water (1:1:1, w/v/v), these values decreased markedly. The material extracted directly into phenol–acetic acid–water (1:1:1, w/v/v) from the wheat flours and dough had weight average molecular weights ranging from 14,000 to 52,000, while that extracted directly into phenol–acetic acid–water (1:1:1, w/v/v) containing 0·2 M sodium bromide had higher molecular weights. The results reported here support earlier evidence that phenol–acetic acid–water (1:1:1, w/v/v) is a very effective solvent for dissociating proteins but that it does not cleave peptide bonds. The results also indicate that intermolecular disulphide bonds are neither present in flour proteins nor formed during the preparation of dough. It is suggested that a more comprehensive study of the physico-chemical properties of proteins can now be made in this solvent.

INTRODUCTION

MIXTURES of phenol, acetic acid and water (1:1:1 and 2:1:1, w/v/v), with and without 0·2 M sodium bromide, have been used to dissolve proteins.[1–5] It has been concluded that these solvents dissociate proteins into their monomeric forms without hydrolysing peptide bonds. It has also been shown that certain enzymes, when recovered from solvents containing phenol, exhibit most of their original activity.[6,7] This suggests that mixtures of phenol, acetic acid and water do not necessarily denature proteins irreversibly.

This paper shows that it is possible to determine the weight average molecular weights, sedimentation coefficients and partial specific volumes of α-chymotrypsin and bovine serum albumin and the weight average molecular weights of insulin and ribonuclease in mixtures of phenol, acetic acid and water.

Since the values for these crystalline proteins agreed well with those obtained by other authors using different methods, the use of phenol–acetic acid–water (1:1:1, w/v/v) as a solvent for dissociating wheat flour proteins was investigated.

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[3] R. L. M. SYNGE, *Metabolism* **13**, 969 (1964).
[4] K. TAKAYAMA, D. H. MACLENNAN, A. TZAGOLOFF and C. D. STONER, *Arch. Biochem. Biophys.* **114**, 223 (1966).
[5] I. BRATTSTEN, R. L. M. SYNGE and W. B. WATT, *Biochem. J.* **97**, 678 (1965).
[6] A. PUSZTAI, *Biochem. J.* **101**, 265 (1966).
[7] A. PUSZTAI. In press.

RESULTS AND DISCUSSION

The results presented in Tables 1 and 2 show that it is possible to determine the weight average molecular weights and sedimentation coefficients of proteins dissolved in mixtures of phenol, acetic acid and water by standard ultracentrifuge methods.[8]

The values obtained for the weight average molecular weights of insulin and ribonuclease in phenol–acetic acid–water (2:1:1, w/v/v) (Table 2) agree well with the minimum molecular weights based on their amino acid compositions.[9,10] These results indicate that the procedures outlined here give valid molecular weight values and are thus in agreement with the observations that peptide bonds are not ruptured in these mixtures.[1–5]

TABLE 1. THE VALUES FOR THE SEDIMENTATION COEFFICIENTS ($S^0_{20,w}$), THE CONSTANTS k^*, AND PARTIAL SPECIFIC VOLUMES ($\bar{V}$) FOR BOVINE SERUM ALBUMIN AND α-CHYMOTRYPSIN IN MIXTURES OF PHENOL, ACETIC ACID AND WATER

Crystalline proteins	Phenol–acetic acid–water (w/v/v)	Sedimentation coefficient ($S^0_{20,w}$ ± S.E.M.)	Constant (k ± S.E.M.)	Partial specific volume, $\bar{V}$ (ml/g)
Bovine serum albumin	2:1:1	3·20 ± 0·17	0·22 ± 0·03	0·71
Bovine serum albumin	1:1:1	3·30 ± 0·15	0·22 ± 0·03	0·75
α-Chymotrypsin	2:1:1	1·43 ± 0·10	0·10 ± 0·03	0·73
α-Chymotrypsin	1:1:1	1·44 ± 0·09	0·04 ± 0·01	0·75

* k was calculated from Schachman's equation[32] as given in the text.

TABLE 2. THE WEIGHT AVERAGE MOLECULAR WEIGHTS OF BOVINE SERUM ALBUMIN, α-CHYMOTRYPSIN, INSULIN AND RIBONUCLEASE IN MIXTURES OF PHENOL, ACETIC ACID AND WATER OR IN 0·15 M SODIUM CHLORIDE

Crystalline proteins	Phenol–acetic acid–water (PAW) (w/v/v) or 0·15 M NaCl	Weight average molecular weight	Weight average molecular weight*
α-Chymotrypsin	1:1:1 (PAW)	17,000	24,500[11,12]
α-Chymotrypsin	2:1:1 (PAW)	17,000	
Insulin	2:1:1 (PAW)	5,500	5,700[9]
Ribonuclease	2:1:1 (PAW)	13,900	13,700[10]
	0·15 M NaCl	14,700	
Bovine serum albumin	1:1:1 (PAW)	17,000	69,000[14]
	0·15 M NaCl	72,000	

* Published values for monomeric forms.

[8] H. K. SCHACHMAN, in *Methods of Enzymology* (edited by S. P. COLOWICK and N. O. KAPLAN), Vol. 4, p. 32. Academic Press, New York (1957).
[9] E. J. HARFENIST and L. C. CRAIG, *J. Am. Chem. Soc.* **74**, 4216 (1952).
[10] C. H. W. HIRS, S. MOORE and W. H. STEIN, *J. Biol. Chem.* **219**, 623 (1956).

The preparation of α-chymotrypsin used in these studies had a weight average molecular weight in mixtures of phenol, acetic acid and water (Table 2) somewhat lower than the known value.[11,12] Pusztai[6] has shown that the physical constants of α-chymotrypsinogen are not altered by contact with phenol and acetic acid provided suitable recovery procedures are used. It is likely therefore that the particular batch of α-chymotrypsin used here had become partly degraded prior to analysis.

Bovine serum albumin has a molecular weight of about 69,000[13,14] in dilute aqueous buffers and is generally considered to be a single polypeptide chain.[14] However, Porter[15] and Richard and Kegeles[16] have presented evidence for the occurrence of subunits in this protein. Foster[17] has postulated that it is composed of four subunits within the same polypeptide chain. Reithel[14] has suggested that these components have a molecular weight of about 16,000 and that the polypeptide may contain atypical bonds and regions of great flexibility. Reichmann and Colvin[18] suggest a molecular weight of 32,000 based on light scattering measurements. Our results (Table 2) are consistent with the view that the molecule is composed of four subunits since the values recorded in Table 2 are 17,000 in phenol–acetic acid–water (1:1:1, w/v/v) and 72,000 in 0·15 M sodium chloride.

The results presented in Table 3 show the aggregates formed by wheat flour proteins in dilute aqueous buffers (extracts A and B) are readily dissociated in phenol–acetic acid–water

TABLE 3. THE WEIGHT AVERAGE MOLECULAR WEIGHTS OF THE MATERIAL IN EXTRACTS OF WHEAT FLOURS AND DOUGH*

Extracts	cv. Gabo flour	cv. Dural flour	Queensland flour	Freeze-dried dough from Queensland flour
A (pyrophosphate extract)	130,000	117,000	127,000	158,000
A_1 (extract A transferred into †PAW, 1:1:1, w/v/v)	28,000	19,000	20,000	23,000
B (acetic acid extract)	225,000	180,000	180,000	155,000
B_1 (extract B transferred into PAW, 1:1:1, w/v/v)	42,000	13,000	46,000	38,500
C (PAW 1:1:1, w/v/v, extract of residue from B)	30,000	14,000	26,500	29,500
D (PAW 1:1:1, w/v/v, extract)	52,000	14,000	48,000	30,000
E (PAW 1:1:1, w/v/v, containing 0·2 M NaBr extract)	73,000	28,000	60,000	48,000

* Details of extractions given in Experimental.
† PAW = Phenol–acetic acid–water.

[11] B. S. HARTLEY, *Nature* **201**, 1284 (1964).
[12] B. W. MATTHEWS, P. B. SIGLER, R. HENDERSON and D. M. BLOW, *Nature* **214**, 652 (1967).
[13] R. A. PHELPS and F. W. PUTNAM, in *The Plasma Proteins* (edited by F. W. PUTNAM), Vol. 1, p. 143. Academic Press, New York (1960).
[14] F. J. REITHEL, *Advan. Protein Chem.* **18**, 123 (1963).
[15] R. R. PORTER, *Biochem. J.* **66**, 677 (1957).
[16] A. J. RICHARD and G. KEGELES, *Arch. Biochem. Biophys.* **80**, 125 (1959).
[17] J. F. FOSTER, in *The Plasma Proteins* (edited by F. W. PUTNAM), Vol. 1, p. 179. Academic Press, New York (1960).
[18] M. E. REICHMANN and J. R. COLVIN, *Can. J. Chem.* **33**, 163 (1955).

(1:1:1, w/v/v) into forms with lower weight average molecular weights (extracts A_1 and B_1) which agree well with previous estimates for the fully dissociated proteins.[19,20] This is further evidence for the validity of the procedures used here for determining weight average molecular weights of proteins and for the stability of peptide bonds in mixtures of phenol, acetic acid and water.

It is of interest that the weight average molecular weights for extracts B_1, C, D and E from cv. Dural are significantly lower than those for cv. Gabo and the other samples. This may be due to genetic difference, since cv. Dural is a tetraploid whereas cv. Gabo and varieties normally used for bread making, are hexaploids.

The weight average molecular weights for extracts C (Table 3) from each of the four samples are much lower than any which have been reported for the unmodified "glutenin" fraction[20–22] and are similar to those of extract A_1 and those obtained for "glutenin" which had been treated with reagents to cleave disulphide bonds.[23] These bonds are rather stable in acid media[24] so it is unlikely that cleavage of intermolecular disulphide linkages was responsible for the dissociation of the aggregates of "glutenin" proteins. The low weight average molecular weights for Queensland flour and the freeze-dried dough prepared from it (Table 3, extract C) provide good evidence that intermolecular disulphide bonds do not occur in flour and are not formed during the preparation of a dough. Therefore, the aggregated proteins in the "glutenin" complex probably interact through non-covalent linkages, namely, electrostatic, hydrogen and hydrophobic bonds.

The weight average molecular weights of the material in extracts D are similar to those for extracts B_1 (Table 3) which contain most of the proteins of the flour. It is apparent that, in extracts E, the inclusion of sodium bromide in the phenol–acetic acid–water mixture has either prevented dissociation or promoted aggregation of these proteins. This effect is contrary to that reported by Brattsten, Synge and Watt[5] who found that sodium bromide promoted the dissociation of nucleic acid–protein complexes prepared from rabbit reticulocytes. Another explanation for the increased molecular weights in phenol–acetic acid–water (1:1:1, w/v/v) containing 0·2 M sodium bromide could be the adsorption of bromide ions by these proteins.

Although phenol and acetic acid may be adsorbed by proteins it is significant that in our experiments with insulin and ribonuclease and with the wheat proteins extracted in dilute pyrophosphate buffer or in dilute acetic acid, their weight average molecular weights in phenol–acetic acid–water (2:1:1, w/v/v) agree well with the lowest values obtained for these proteins in aqueous systems.[19,20,25,26] The results for insulin and ribonuclease are also similar to the minimal molecular weight determinations based on the amino acid composition of these proteins.[9,10] It is also relevant that the values for the partial specific volume of bovine serum albumin and of α-chymotrypsin in phenol–acetic acid–water (1:1:1, w/v/v) do not differ significantly from values obtained in aqueous buffers.[27,28] Thus these results indicate that a

[19] B. SULLIVAN, *Cereal Sci. Today* **10**, 338 (1965).
[20] Y. V. WU, J. E. CLUSKEY and K. R. SEXSON, *Biochem. Biophys. Acta* **133**, 83 (1967).
[21] R. W. JONES, G. E. BABCOCK, N. W. TAYLOR and F. R. SENTI, *Arch. Biochem. Biophys.* **94**, 483 (1961).
[22] R. W. JONES, G. E. BABCOCK, N. W. TAYLOR and F. R. SENTI, *Arch. Biochem. Biophys.* **104**, 527 (1964).
[23] H. C. NIELSEN, G. E. BABCOCK and F. R. SENTI, *Arch. Biochem. Biophys.* **96**, 252 (1962).
[24] R. CECIL and J. R. MCPHEE, *Advan. Protein Chem.* **14**, 255 (1959).
[25] D. F. WAUGH, *Advan. Protein Chem.* **9**, 325 (1954).
[26] R. TRAUTMAN and C. F. CRAMPTON, *J. Am. Chem. Soc.* **81**, 4036 (1959).
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[28] G. W. SCHWERT, *J. Biol. Chem.* **179**, 655 (1949).

correct determination of anhydrous weight average molecular weight can be achieved under conditions where solvation by phenol and acetic acid may occur.[29] It is clear that further information is required on the behaviour of macromolecules in these solvent systems.

EXPERIMENTAL

Materials

Crystalline proteins were purchased as follows: Bovine pancreatic α-chymotrypsin, bovine insulin from Sigma Chemical Co., St. Louis, Missouri; bovine serum albumin (electrophoretically homogeneous) from Calbiochem, Los Angeles, California; bovine pancreatic ribonuclease from Worthington Biochemical Corporation, Freehold, New Jersey. These were used without further purification.

Protein fractions from flours prepared from grain of *Triticum vulgare* cv. Gabo and *T. durum* cv. Dural, from a commercial sample of baking flour from Queensland and from a freeze-dried sample of dough prepared from it were also examined. The latter two samples were supplied by Mr. M. V. Tracey of the Wheat Research Unit, C.S.I.R.O., North Ryde, N.S.W.

The phenol and acetic acid were redistilled before use.

Extraction of Proteins from Wheat Flour and Dough

Portions of the flour and dough samples were extracted by the procedure of Graham, *et al.*[30] except that phenol–acetic acid–water (PAW) (1:1:1, w/v/v) was used instead of 0·1 N NaOH. In this procedure, the material was extracted with *n*-butanol saturated with water, then with three portions of 0·01 M sodium pyrophosphate buffer (pH 7·4). The first of the three pyrophosphate extracts was labelled A. The material was then extracted with three portions of 0·05 N acetic acid and the first of these extracts was labelled B. The residue was extracted with three portions of PAW (1:1:1, w/v/v); the first one was labelled C. The transfer of the material in Extracts A and B into PAW (1:1:1, w/v/v) by dialysis (see below) gave extracts A_1 and B_1.

Portions of the flour and dough samples were also extracted directly with PAW (1:1:1, w/v/v) (extracts D) and with the same solvent containing 0·2 M NaBr (extracts E). Extracts D and E contained at least 96 per cent of the total nitrogen. The inclusion of NaBr in the solvent did not significantly affect the total amounts of nitrogen or dry matter extracted from these samples.

Treatment of Extracts Prior to Centrifuging

The extracts were dialysed for at least 12 hr against 10 volumes of the same solvent in which they were extracted. Three changes of the solvent were made when an extract was being dialysed into a different solvent system. The final diffusate, which was in equilibrium with the extract in the dialysing bag, was used in the reference sector of the ultracentrifuge cell. Protein concentrations were adjusted to between 5 and 10 mg/ml for analysis, using the diffusate if the adjustment was done after dialysis.

Ultracentrifuge Studies

The sedimentation coefficients and molecular weights were determined in a Beckman Model E analytical ultracentrifuge equipped with a Schlieren optical system and a temperature control unit (RTIC). All measurements were made at room temperature (18–23°). The corrosive nature of solvents containing phenol and acetic acid made it necessary to use cells fitted with aluminium centre pieces lined with Kel F.

Ostwald viscometers, made to British Standard 188, were used to determine viscosities; partial specific volumes were measured in pycnometers. Both procedures are described by Schachman.[8] The values for the partial specific volume of bovine serum albumin and α-chymotrypsin in mixtures of phenol, acetic acid and water are shown in Table 1. Since these values agreed well with those obtained in dilute aqueous buffers[27,28] the partial specific volumes of the flour proteins, in mixtures of phenol, acetic acid and water and in aqueous buffers were assumed to be 0·73 ml/g.[8] Partial specific volumes for insulin and ribonuclease were taken to be 0·72 ml/g[31] and 0·71 ml/g.[8]

The sedimentation coefficients were determined by the method of Schachman.[8] Single sector centre pieces were used and the rotor operated at 59,780 rev/min. The k values were calculated from the following equation:[32]

$$S_{20,\,w}(1=c)k+S_{20,\,w}$$

for values of c between 1 and 10 mg/ml. The proteins migrated as single symmetrical boundaries.

The weight average molecular weights were determined by the approach to equilibrium technique of Archibald[33] as described by Schachman[8] using a double-sector centre piece. The synthetic boundary centre

[29] H. K. SCHACHMAN, in *Ultracentrifugation in Biochemistry*, p. 219. Academic Press, New York (1959).
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piece was of the double-sector type with capillary interconnections. A window with a 1° positive wedge was used on the synthetic boundary cell. This made it possible to run both cells at the same time.

The values reported here were those measured at the upper meniscus. Measurements at the lower meniscus could not be made because the oils, FC43 and DC550, were slightly soluble in the solvents containing phenol. Consequently, oils were not used in these experiments.

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